STUDIES ON INHIBITION, ATTENUATION, AND REJUVENATION OF BACILLUS COLI.*

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THERE has been much discussion and uncertainty as to the delicacy and relative value of dextrose broth[1] and lactose bile[2,3,4] in testing water of fairly good quality.[5,6,7] The present investigation was undertaken with a view to throwing light upon this subject.

Comparison has been made between plain broth, dextrose broth, lactose broth, lactose bile, and other liquid media under greatly varying conditions, artificial and natural. In the case of plain broth, 1 c.c. was transplanted at the end of 24 hours into lactose bile and in some series into dextrose broth. In other series dextrose broth was transplanted into lactose bile after 24 or 48 hours. In a few experiments bile was transplanted into bile.

EXPERIMENTS UNDER ARTIFICIAL CONDITIONS.

Two sets of experiments were devised and carried out in different years with practically the same results. The object was to ascertain whether dextrose broth would show gas formation in higher dilution than would lactose bile. The first series was carried out with well water, the second with surface water. In each case, the water was not sterilized but was tested with both dextrose broth and lactose bile to prove the absence of gas-forming bacteria. The surface water was in addition incubated at 37° C. for two days before testing for gas formers. A pure culture of B. coli was then introduced into the bottles of water and the samples kept at three temperatures, 37° C., 20° C., and 8° C. Daily tests were made upon each sample with dextrose broth and with lactose bile in quantities of 10 c.c., 1 c.c., 0.1 c.c., 0.01 c.c., etc., using a sufficient number of dilutions by tenths always to obtain the zero point, until gas formation was no longer obtained in 10 c.c. of the water. The accompanying charts, A and B, show the results of these experiments.

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As no gas formers were originally present, the formation of any amount of gas, no matter how small, in the dextrose broth indicated B. coli. Unsterilized water was used in order to approach natural conditions and allow of interference with the tests for B. coli by the water bacteria present. The tests were planned to show also any

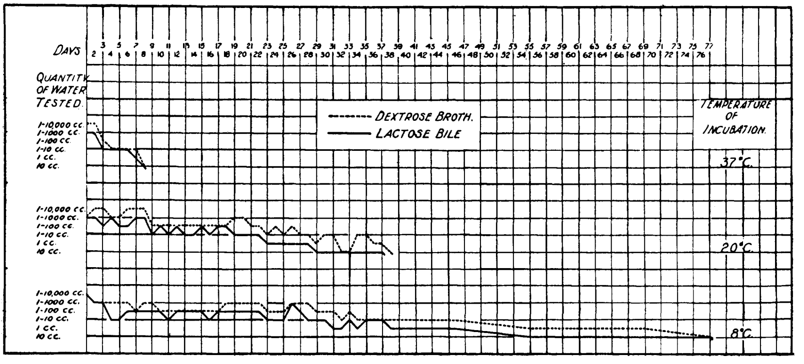

CHART 1.—Comparison, made with dextrose broth and lactose bile, of tests for B. coli inoculated into unsterilized well water.

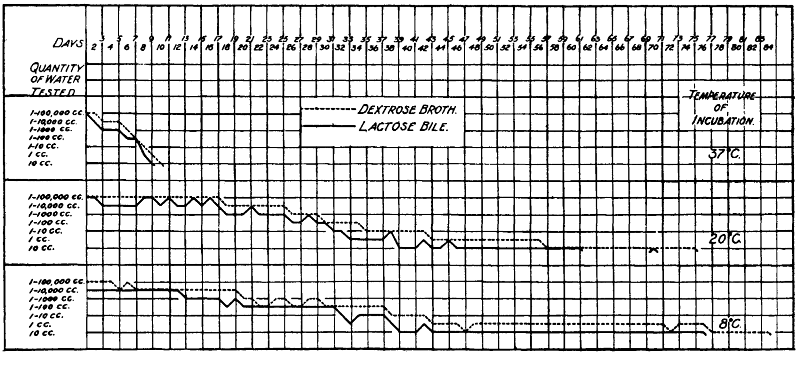

CHART 2.—Comparison, made with dextrose broth and lactose bile, of tests for B. coli inoculated into unsterilized surface water.

difference that might exist between dextrose broth and lactose bile in indicating B. coli when vigorous and when attenuated. The experiments likewise serve to demonstrate the viability of B. coli at the three temperatures and under the existent conditions.

Viability.—It will be seen from the charts that the B. coli quickly died out in the samples kept at 37° C., lasting only 8 to 10 days.

At 20° C. and 8° C., B. coli lived much longer, 38 to 75 days at 20° C. and 77 to 84 days at 8° C. Even then its presence was detected by a more delicate medium, liver broth,[8] recently devised in this laboratory.

It is important to know in regard to samples of water which have necessarily been some time in transit whether the test for B. coli is trustworthy as evidence of the presence and amount of pollution. The charts seem to indicate that samples shipped without ice, unless they should become considerably warmed in transit, may yield good evidence even if a week old.[9]

Bacteria present.—In the first set of experiments (Chart 1), the samples were plated daily in litmus-lactose-agar and in gelatin. The well water originally showed 12 bacteria per c.c. on gelatin. After inoculation with B. coli, litmus-lactose-agar gave counts per c.c. for the first few days, before the bacteria had become attenuated, as follows:

Sample incubated at 37° C.—14,000, 2,000, 200; at 20° C. 6,000, 8,000, 5,900, 3,200; at 8° C.—80,000, 4,000, 3,700, 900, 1,200.

Comparing these counts with the dilutions showing gas formation in dextrose broth and lactose bile it will be seen that the number of bacteria giving a test are as follows:

Dextrose broth, series at 37° C.—14, 1, 2; at 20° C.—6, 1, 1, 3; at 8° C.—8, 4, 4, 1, 1; average 4.

Lactose bile, series at 37° C.—140, 2, 20; at 20° C.—6, 8, 59, 3; at 8° C.—8, 4, 4, 90, 120; average 39.

While the colon bacilli were gradually dying out, the water bacteria were rapidly increasing, and also new species were introduced from time to time because of the daily slight exposure of the bottles to the air in testing. In the series at 37° C. the agar plates showed 30,000 on the fourth day, due to water forms which grow at 37° C. In a few days all bacteria had disappeared from this sample with the exception of a few bacteria growing on gelatin at 20° C. In the series at 20° C. the water bacteria as shown by gelatin increased to a maximum of 242,000 per c.c. on the fifth day, dropped to 700 on the ninth day, rose to 66,000 on the tenth day and then fluctuated between 1,500 and 13,000 for the remainder of the time. At the end they were slowly increasing and had reached 4,000. In the

series at 8° C. the water bacteria reached 10,000 on the seventh day, dropped to 900 on the ninth day, reached a maximum of 20,000 on the tenth day, increased from 6,000 on the eleventh to 13,000 on the sixteenth and from 3,500 on the seventeenth to 17,000 on the twenty-fourth day. B. liquifaciens fluorescens made its first appearance on the nineteenth day and continued thereafter. From the twenty-fifth to the twenty-eighth day there were about 12,000 bacteria present, from the thirty-first to the thirty-sixth 5,000, thirty-seventh to fortieth 15,000, decreasing to 3,000 on the forty-fourth day. There was no very apparent relationship between the fluctuations of water bacteria and the tests for B. coli. Occasionally a sudden increase in the water bacteria was accompanied by a corresponding decrease in the test for B. coli.

Dextrose broth.—As a presumptive test for B. coli, dextrose broth has been limited to certain empirical rules, namely, gas production 25 per cent to 70 per cent and carbon dioxide 25 per cent to 40 per cent. Extended experience has shown this test to be very unsatisfactory for several reasons:[7, 10, 11, 12, 13] (1) several other species will give the formula; (2) many other species produce gas in dextrose broth;[10] (3) other bacteria interfere with the test, causing numerous anomalies.[12]

A most striking feature of the above series of experiments was the complete failure of the formula[7, 13] in the presence of no other gas former than B. coli. This was probably due to interference of the water bacteria. Out of 818 tests which showed gas formation, only 474, or 58 per cent, met the formula. Out of 259 sets of dilutions only 62, or 24 per cent, failed to show one or more anomalies.

Lactose bile.—In contrast to dextrose broth, the bile gave 599 positive tests for B. coli, i.e., over 25 per cent total gas within three days. Only three tests gave gas which did not reach 25 per cent. There was not a single anomaly, i.e., each set of dilutions showed a consecutive series of positive tests.

The rapidity with which the gas appeared in the bile also gave some idea of the degree of attenuation of the B. coli. A study of the details of the series of experiments represented in Chart 1 brought out the following features:

For six days every test in the bile was positive in 24 hours. After

the sixth day only two tests became positive in 24 hours, one on the eighth day and one on the twenty-fifth day. On the ninth day occurred the first test that required three days to develop and that was the highest dilution of a set. On the twelfth day three-day tests were frequent. On the sixteenth day for the first time every test required three days, although two-day tests were frequent to the end. On the thirty-first and thirty-eighth days occurred the three tests which failed to show 25 per cent gas in three days.

Comparative delicacy.—The series of experiments represented in Chart 1 were carried out with the lactose bile as first described. In the series of Chart 2 lactose bile with 1 per cent peptone[14] added was used, since it had been learned in the meantime that the addition of peptone hastened the production of gas and increased its quantity, rendering the test considerably more delicate. It may be stated in passing that the addition of Nährstoff Heyden[15] and of fresh meat infusion to lactose bile were tried, but failed to improve its delicacy in any respect. Peptone alone improved it. In the present experiments the results did not show the usual improvement of lactose bile with peptone over lactose bile except in a very slight degree.

A comparison between dextrose broth, considering any gas formation as a positive test, since B. coli only was present, and the test as given by lactose bile and by lactose bile with peptone is given as follows:

Dextrose broth gave positive tests more delicately than

	Lactose Bile		Lactose Bile with Peptone	
Two dilutions* greater	14 times	—11%	10 times	— 6%
One " "	81 "	66 "	124 "	73 "
No " "	28 "	23 "	35 "	21 "
	123		169	

* All dilutions are in tenths.

Allowing double weight for two dilutions the dextrose broth was one dilution ahead of lactose bile 109 times out of 123, i.e., 89 per cent, and similarly ahead of lactose bile with peptone 144 times out of 169, i.e., 85 per cent. Considered from another standpoint it required about 10 bacilli to produce a test in lactose bile for every 1 bacillus necessary to produce gas in dextrose broth under the conditions of these experiments. This was due to the inhibitive action

of the bile salts, since lactose broth showed gas in as high dilution as did the dextrose broth when comparisons were made, i.e., the sugar, lactose, was not the inhibiting agent.

Rejuvenation tests.—Toward the end of these experiments, while B. coli was attenuated, additional comparisons were made by planting dilutions into plain broth and transplanting into lactose bile at the end of twenty-four hours. Again comparisons were made using dextrose broth made with fresh beef infusion, instead of Liebig's beef extract as used in the regular dextrose broth. The results of these comparisons are seen as follows:

Rejuvenation in plain broth gave a test for B. coli:

One dilution greater than dextrose broth (Liebig's extract)	8	times
No " " " " " "	9	"
One " less " " " " "	3	"
	20	
One dilution greater than dextrose broth (beef infusion)	1	time
No " " " " " "	16	"
One " less " " " " "	3	"
	20	
Three dilutions greater than lactose bile	2	times
Two " " " " "	4	"
One " " " " "	11	"
No " " " " "	3	"
	20	

The results of these tests were averaged by multiplying a three-dilution advantage by three, a two-dilution advantage by two, adding the single dilution advantage and subtracting the dilution disadvantage. The results thus compared showed that rejuvenation in plain broth in these twenty experiments gave a test on an average $1\frac{1}{4}$-dilution higher than lactose bile, $\frac{1}{4}$-dilution higher than dextrose broth (Liebig's extract), and $\frac{1}{10}$ dilution less than dextrose broth (fresh beef infusion). It is apparent that the difference between plain broth and dextrose broth was due to the presence and absence of fresh meat infusion and not to the presence of sugar. Hence by planting dilutions of water into dextrose broth made with fresh meat infusion and, as soon as gas formation appears, transplanting into lactose bile, a larger number of tests for B. coli may be obtained than by using lactose bile alone. This excess B. coli is usually,

however, attenuated and of less importance than vigorous B. coli which indicates fresh contamination.

EXPERIMENTS WITH ROUTINE SAMPLES OF WATER UNDER NATURAL CONDITIONS.

The previous experiments were conceived for the purpose of keeping strict control over all conditions with an exact knowledge of the gas former present. Altho the samples were unsterilized, thus allowing for the presence of large numbers of water bacteria, yet the experiments could not fail to be artificial in character.

In order to supplement and test under a wider range of natural conditions the information gained, daily comparisons were made upon Manhattan and Brooklyn waters between dextrose broth and lactose bile in one year and between lactose bile, lactose bile with peptone, and dextrose broth transplanted to lactose bile with peptone, in another year, during the seasons when B. coli is prevalent. Some 60 comparisons were also made with plain broth transplanted to bile.

The results of comparisons upon the waters of the two boroughs have been kept separate, and contrary to expectation the bile appeared to the best advantage in testing the water supplied to Manhattan. This was true in every comparison of both years. The Manhattan supply has long storage and B. coli should be more attenuated than in the Brooklyn water. The reason for these results probably rests with a difference in the water bacteria present which interfere with the gas production or growth of B. coli.

The following table gives the results of comparison for the first year between dextrose broth and lactose bile. With the dextrose

TABLE I.

		Positive Presumptive Tests for B. Coli		Total Gas Formers
		Lactose Bile	Dextrose Broth (formula)	Dextrose Broth 5% Gas and Over
Manhattan supply (399 samples)	0.1 c.c.	16— 4%	20— 5%	139—35%
	1.0 "	52—13 "	47—12 "	246—62 "
	10.0 "	178—45 "	105—26 "	354—89 "
Brooklyn supply (933 samples)	0.1 "	26— 3 "	82— 9 "	285—31 "
	1.0 "	119—13 "	221—24 "	642—69 "
	10.0 "	418—45 "	408—44 "	868—93 "
Totals (1,332 samples)	0.1 "	42— 3 "	102— 8 "	424—32 "
	1.0 "	171—13 "	268—20 "	888—67 "
	10.0 "	596—45 "	513—39 "	1,222—92 "

broth the formula was adhered to as a test for B. coli. There are also included in the table the tests in dextrose broth showing 5 per cent or more of gas, which gives a good idea of the number of gas formers present, the greater part of which are not B. coli, in surface waters of good sanitary quality.

Anomalies.[12]—The anomalies shown by the use of dextrose broth and the formula are well illustrated in the following table:

TABLE 2.

	Form of Anomaly			Manhattan Supply	Brooklyn Supply	Total
	0.1 c.c.	1.0 c.c.	10.0 c.c.			
Dextrose broth	+	0	0	10	26	36
	+	+	0	3	14	17
	+	0	+	4	20	24
	0	+	0	15	83	98
Total				32	143	175
Lactose bile	+	0	0	1	1	2
	+	+	0	0	0	0
	+	0	+	4	2	6
	0	+	0	6	7	13
Total				11	10	21

There is certainly much greater interference with proper gas production in dextrose broth than in lactose bile.

The second year's comparisons give a better idea of the true relative value of the test media, since transplanting to bile established very closely the actual amount of B. coli present. The following table gives the number and percentages of positive tests for B. coli obtained by the different media, and also in the last column the number and percentages of total gas formers present.

The first two columns show no very great difference between the lactose bile and the dextrose broth formula, there being a slight advantage for the bile.

The second and third columns illustrate the great improvement caused by adding peptone to the lactose bile.

The third, fourth, and fifth columns prove that the lactose bile with peptone produces in practice substantially the same results as rejuvenation in dextrose broth, made with Liebig's extract, combined with transplanting to bile. With Manhattan water the bile decidedly had the advantage.

The last column shows again that an excess of gas formers, not B. coli, may be present in water of good quality.

TABLE 3.

		Dextrose Broth (formula)	Lactose Bile	Lactose Bile with Peptone	Dextrose Broth, 5% Gas and Over, Transplanted to Bile	Duplicate Bile Tests	Dextrose Broth 5% Gas and Over
Manhattan supply (85 tests)	0.1 c.c. ...	0— 0.0%	1— 1.2%	1— 1.2%	0— 0.0%	2— 2.4%	0— 0.0%
	1.0 " ...	6— 7.1 "	5— 5.9 "	13—15.4 "	4— 4.7 "	15—17.7 "	14—16.5 "
	10.0 " ...	24—28.2 "	31—36.5 "	54—63.5 "	48—56.5 "	60—70.6 "	59—69.4 "
Brooklyn supply (160 tests)	0.1 " ...	1— 0.6 "	1— 0.6 "	1— 0.6 "	2— 1.3 "	2— 1.3 "	15— 9.4 "
	1.0 " ...	11— 6.9 "	15— 9.4 "	25—15.6 "	25—15.6 "	31—19.4 "	62—38.8 "
	10.0 " ...	51—31.9 "	63—39.4 "	72—45.0 "	93—58.1 "	88—55.0 "	121—75.6 "
Total (245 tests)	0.1 " ...	1— 0.4 "	2— 0.8 "	2— 0.8 "	2— 0.8 "	4— 1.7 "	15— 6.3 "
	1.0 " ...	17— 7.0 "	20— 8.2 "	38—15.5 "	29—12.0 "	46—18.8 "	76—31.4 "
	10.0 " ...	75—30.7 "	94—38.4 "	126—51.2 "	141—57.6 "	148—60.4 "	180—73.5 "

In the experiments conducted under artificial conditions the number of formula tests for B. coli was 58 per cent of the number of tests showing gas formation. In the above table it is interesting to note that the number of formula tests is 54 per cent of the number of tests for B. coli shown in dextrose broth by gas formation corroborated by transplanting to bile.

Rejuvenation tests.—About 60 rejuvenation tests were also made on these waters, planting in plain broth and transplanting to bile. The results are shown in the following table:

TABLE 4.

		Lactose Bile with Peptone	Plain Broth Transplanted to Bile
Manhattan supply (19 samples)	0.1 c.c.	1— 5.3%	0— 0.0%
	1.0 "	2—10.5 "	1— 5.3 "
	10.0 "	6—31.6 "	6—31.6 "
Brooklyn supply (38 samples)	0.1 "	1— 2.6 "	2— 5.3 "
	1.0 "	8—21.1 "	12—31.6 "
	10.0 "	27—71.1 "	32—84.2 "
Total (57 samples)	0.1 "	2— 3.5 "	2— 3.5 "
	10.0 "	10—17.6 "	13—22.8 "
	10.0 "	33—57.9 "	38—66.7 "

Rejuvenation in plain broth showed a slight advantage on the Brooklyn water, probably due to the fresh beef infusion in its composition.

The same samples were also used in a comparison between gas

formation in dextrose broth, made with Liebig's extract, before and after rejuvenation in plain broth with the following results:

TABLE 5.

		Dextrose Broth (Gas Formation)	Plain Broth Transplanted to Dextrose Broth
Manhattan supply (18 samples)	0.1 c.c.	9— 50%	13— 72%
	1.0 "	15— 83 "	15— 83 "
	10.0 "	17— 94 "	18—100 "
Brooklyn supply (37 samples)	0.1 "	7— 19 "	14— 38 "
	1.0 "	30— 81 "	31— 84 "
	10.0 "	37—100 "	37—100 "
Total (55 samples)	0.1 "	16— 29 "	27— 49 "
	1.0 "	45— 82 "	46— 84 "
	10.0 "	54— 98 "	55—100 "

There was considerable improvement after rejuvenation, showing the necessity for fresh beef infusion in dextrose broth, as previously indicated. An abundance of gas formers was again shown in water containing comparatively very little B. coli.

Transplanting.—Rather uncertain results may sometimes follow transplanting, so that as a routine procedure it is not to be highly recommended. This is due partly to interference of bacteria and largely to errors of inoculation.

The following table has been compiled from a large number of transplantings (360):

TABLE 6.

	B. coli Present	Gas in Dextrose Broth	Formula Correct	Transplanted to Bile	Number of Tests
A	+	+	+	+	40
B	+	+	o	+	72
C	+	o	o	+	9
					121
D	+	+	+	o	6
E	+	+	o	o	17
F	+	o	o	o	9
					32
G	o	+	+	o	21
H	o	+	o	o	99
					120
		Lactose Bile Positive Test			
I	+	+	..	+	15
J	+	o	..	+	11
K	+	+	..	o	6
L	o	o	..	o	55
					87

Among other things the table shows that a formula test in dextrose broth may be caused by other bacteria than B. coli in quite a percentage of cases (see G), and on the other hand that transplanting may sometimes fail to show B. coli altho present (see D, E, F, and K). Several times, transplanting to bile brought up a test when originally no gas had appeared (see C and J).

CONCLUSIONS.

1. Experiments conducted under carefully regulated conditions have shown that the bile salts in lactose bile cause an appreciable degree of inhibition in the development of B. coli.

2. This inhibition increases with attenuation.

3. Rejuvenation in suitable media followed by transplanting to lactose bile will sometimes prove the presence of B. coli, usually attenuated, not shown by the lactose bile in direct tests.

4. In actual practice covering hundreds of samples of Manhattan and Brooklyn waters, the lactose bile, made with the addition of 1 per cent peptone, has been shown to practically equal the results obtained by rejuvenation in dextrose broth, made with Liebig's extract, followed by transplanting to lactose bile.

5. If dextrose broth is continued in use, it should be made with fresh beef infusion, since it is then more delicate than when made with Liebig's extract. The formula test is of but little practical value and transplanting to lactose bile should be made as soon as gas appears.

6. Gas formation in lactose bile after transplanting from the plain broth or dextrose broth is not always certain, even when B. coli is present, on account of interfering growths in the original medium.

7. Lactose bile gives more reliable *presumptive* tests for B. coli than any other known medium,[16, 17, 18] including aesculin broth.[19, 20]

8. Other species of bacteria cause much less interference with gas formation of B. coli in lactose bile than in other media.

9. To rejuvenate and transplant seems too laborious and uncertain in routine work. The information gained assists more especially in interpreting the results obtained with lactose bile. Lactose bile makes a distinction between recent and distant contamination, hence gives better evidence of the actual relative sanitary quality of a water.

10. The use of lactose bile as a step in the separation of B. typhosus[21] from water adds yet weightier reasons for its direct employment in the examination of water.

REFERENCES.

1. SMITH. *Annual Report State Board of Health of New York*, 1892, p. 712.
2. JACKSON. *Biological Studies by the Pupils of William Thompson Sedgwick*, The University of Chicago Press, 1906.
3. ———. *Jour. Infect. Dis.*, 1907, Supplement 3, p. 30; also *Am. Pub. Health Assoc. Papers and Reports*, 1906, 32, Part 2, p. 30.
4. ———. *Amer. Jour. Pub. Hyg.*, 1908, 18, p. 16; also *Amer. Pub. Health Assoc. Papers and Reports*, 1907, 33, Part 2, p. 101.
5. PRESCOTT AND WINSLOW. *Amer. Jour. Pub. Hyg.*, 18, p. 19; also *Amer. Pub. Health Assoc. Papers and Reports*, 1907, 33, Part 2, p. 128.
6. SAWIN. *Jour. Infect. Dis.*, 1907, Supplement 3, p. 33; also *Amer. Pub. Health Assoc. Papers and Reports*, 1906, 32, Part 2, p. 33.
7. LONGLEY AND BATON. *Jour. Infect. Dis.*, 1907, 4, p. 397.
8. JACKSON AND MUER. Paper presented to Laboratory Section, Amer. Pub. Health Assoc. Convention, 1909.
9. JORDAN AND IRONS. *Amer. Pub. Health Assoc. Papers and Reports*, 1899, 25, p. 566.
10. CLARK AND GAGE. *Report Mass. State Board of Health*, 1902, p. 262.
11. WINSLOW AND HUNNEWELL. *Jour. Med. Res.*, 1902, 8, p. 502.
12. WHIPPLE. *Tech. Quarterly*, 1903, 16, p. 18.
13. FULLER AND FERGUSON. *Jour. Infect. Dis.*, 1905, Supplement 2, p. 142.
14. JACKSON. "Progress Report of the Committee on Standard Methods for the Bacterial Examination of Water and Sewage," *Amer. Jour. Pub. Hyg.*, 18, p. 413.
15. HESSE AND NIEDNER. *Ztschr. f. Hyg.*, 1898, 29, p. 454.
16. IRONS. *Jour. Hyg.*, 1902, 2, p. 314.
17. GAGE AND PHELPS. *Trans. Amer. Pub. Health Assoc.*, 1902, 28, p. 402.
18. PRESCOTT AND WINSLOW. *Elements of Water Bacteriology*, New York, 1908, pp. 149–51.
19. HARRISON AND VAN DER LECK. *Amer. Jour. Pub. Hyg.*, 1909, 19, p. 557.
20. ———. Paper to be published from this laboratory. Other species, some not gas formers, give the aesculin test.
21. JACKSON AND MELIA. *Jour. Infect. Dis.*, 1909, 6, pp. 194–204; also *Amer. Jour. Pub. Hyg.*, 1909, 19, p. 83.